The integration of Aspergillus niger and Saccharomyces cerevisiae co-culture enables the production of bioethanol from waste potatoes

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Abstract: **Bioethanol was a type of bioenergy that was highly effective and environmentally friendly. Raw materials, including agricultural crops and waste materials from households or industries, were used as carbon sources to create ethanol. In this work, ethanol was produced from waste potato peel. The co-culture method was employed, using *Aspergillus niger* and *Saccharomyces cerevisiae* to convert the waste into valuable products. The process, known as simultaneous saccharification and fermentation (SSF), combined enzyme degradation and microbial fermentation into a single stage. SSF enhanced the output and efficiency of bioethanol production by integrating fermentation with starch hydrolysis, reducing energy requirements and processing time, which also made bioethanol production more profitable. *Aspergillus niger* broke down cellulose and starch into fermentable sugars. It generated the enzymes glucoamylase and alpha-amylase, which converted potato starch into glucose. During fermentation, *Saccharomyces cerevisiae* converted glucose from potato starch into bioethanol, maintaining the necessary biological, chemical, and physical parameters for high production, with an optimal pH of 4.5–6.5 and an optimal temperature of 28–32°C. *Saccharomyces cerevisiae* could tolerate high ethanol concentrations, leading to effective bioethanol production. The recovery process for bioethanol involved separating and purifying the bioethanol from the fermented broth. Initially, leftover biomass was removed through solid-liquid separation methods like centrifugation or filtration. The ethanol was then concentrated from the liquid stream through a distillation process. Bioethanol of 95% purity met specific standards, ensuring its suitability for various applications. This high-purity bioethanol contained 80% or more ethanol, with 15% or less water content, and was free from harmful impurities.**

***Keywords: Aspergillus niger*, Bioethanol, *Saccharomyces cerevisiae*, Saccharification-fermentation Waste Potatoes.**

INTRODUCTION

The increasing reliance on fossil fuels presented two major concerns: environmental degradation and the depletion of non-renewable resources. Fossil fuels contributed significantly to greenhouse gas emissions, leading to climate change and air pollution. Additionally, petroleum reserves were being depleted at an alarming rate, making it imperative to find sustainable energy alternatives. Bioethanol emerged as a promising renewable energy source due to its potential to replace fossil fuels in transportation and industrial applications. The purpose of this project was to develop an efficient and cost-effective method for producing bioethanol from renewable feedstocks. Specifically, it focused on the fermentation of potato waste to generate bioethanol, a viable alternative to fossil fuels. The key objectives of this project included reducing dependence on petroleum-based fuels, investigating the efficiency of microorganisms in bioethanol production, optimizing fermentation and enzymatic hydrolysis processes, and promoting environmental sustainability.

The bioethanol production process involved several key steps. First, the potato waste underwent physical and chemical pretreatment to break down complex carbohydrates. Then, enzymes such as alpha-amylase and glucoamylase catalyzed the breakdown of starch into simple sugars. Next, the resulting sugars were converted into ethanol and carbon dioxide through fermentation. Finally, the fermented broth was subjected to distillation, which separated ethanol from water and other components. [1]-[4]

Aspergillus niger and *Saccharomyces cerevisiae* were two microorganisms that played crucial roles in the bioethanol production process. *Aspergillus niger*, a filamentous fungus, produced enzymes that broke down complex carbohydrates in potato waste into simple sugars. This process, known as

saccharification, was essential for converting potato waste into a sugar-rich hydrolysate that could be fermented by *Saccharomyces cerevisiae*.

The enzymes produced by *Aspergillus niger*, such as alpha-amylase and glucoamylase, hydrolyzed starches and other complex carbohydrates into fermentable sugars. This increased the sugar yield from potato waste, making the bioethanol production process more efficient. *Aspergillus niger* enzymes also improved the accessibility of sugars for fermentation, leading to higher bioethanol yields.

Saccharomyces cerevisiae, also known as baker's yeast, was a unicellular fungus that fermented the sugar-rich hydrolysate produced by *Aspergillus niger*. This microorganism was known for its high ethanol yield and tolerance to fermentation conditions, making it an ideal microorganism for bioethanol production. *Saccharomyces cerevisiae* fermentation efficiency was optimized by controlling factors such as temperature, pH, and substrate concentration. The combination of *Aspergillus niger* and *Saccharomyces cerevisiae* created a synergistic relationship that enhanced the overall efficiency of the bioethanol production process. *Aspergillus niger* enzymes broke down complex carbohydrates into simple sugars, which were then fermented by *Saccharomyces cerevisiae* to produce bioethanol. By understanding the roles of these two microbes, their interactions were optimized, leading to a more efficient bioethanol production process. [5]-[11]

A series of tests were conducted to analyze the bioethanol sample, including the iodine test to detect starch or carbohydrates and the sulfuric acid test to identify carbohydrates or sugars. The boiling point test was also performed to determine the boiling point of the bioethanol sample. Furthermore, a pH test, density test, and IR test were conducted to assess the acidity/basicity, density, and molecular structure of the sample, respectively. Additionally, an antimicrobial activity test was carried out to evaluate the antimicrobial properties of the bioethanol sample, and a validation test was performed to verify the accuracy and reliability of the analytical methods used.

These tests were performed to ensure the quality and purity of the bioethanol produced. They helped detect contaminants, determine physical properties, and verify the presence of desired compounds. By conducting these tests, it was confirmed that the bioethanol met the required standards for use as a fuel or solvent. Moreover, the tests ensured that the bioethanol was safe for use and did not pose any environmental risks. Ultimately, the quality and reliability of the bioethanol were guaranteed through the successful completion of these tests. [12]-[17]

These tests were performed to ensure the quality and purity of bioethanol. They detected contaminants, determined physical properties, and verified the presence of desired compounds. The tests helped to confirm that the bioethanol met the required standards for use as a fuel or solvent. They also ensured the bioethanol was safe for use and did not pose any environmental risks. By performing these tests, the quality and reliability of the bioethanol were guaranteed.

Utilized Agricultural Waste: It converted agricultural waste into valuable resources, reducing waste disposal issues. Supported Rural Development: Bioethanol production created jobs, stimulated local economies, and enhanced rural livelihoods. Enhanced Food Security: By using non-food crops and waste materials, it ensured food security and supported sustainable agriculture. Chemical By-Production: Bioethanol produced valuable chemicals like ethylene and acetic acid, supporting industrial applications. Pharmaceutical Applications: It provided solvents and intermediates for pharmaceutical manufacturing, enhancing healthcare. Cosmetics and Personal Care: Bioethanol was used in cosmetics and personal care products, ensuring safety and efficacy and supporting consumer well-being. Bioethanol represented a sustainable and eco-friendly alternative to fossil fuels, addressing both environmental and economic concerns. By utilizing microbial fermentation processes with *Aspergillus niger* and *Saccharomyces cerevisiae*, this project aimed to enhance the efficiency of bioethanol production from potato waste. [31],[33],[36]

METHODOLOGY

Strain Preparation

a. For the *Aspergillus niger*

The spoiled onion with black growth was collected from the farm. A suspension was prepared by mixing a small portion of the spoiled onion in sterile distilled water. The PDA plates were prepared by pouring the sterilized PDA medium into petri plates. The suspension was streaked onto the PDA plates using a sterile inoculation loop. The plates were incubated at

25-30°C for 3-5 days to allow the growth of microorganisms. Colony morphology observation: The plates were observed for colony morphology, such as shape, size, colour, and texture. Performed Lactophenol Cotton Blue Staining Performed Sugar Test (Sucrose-Maltose)

b. For the *Saccharomyces cerevisiae*

The bread sample was collected from a local bakery and stored and transported to the laboratory. A suspension was then prepared by mixing 1g of bread with 9ml of sterile water. The yeast was isolated by preparing serial dilutions up to 10^-5, which were then plated on Yeast Peptone Dextrose Agar (YPD) plates. The plates were incubated at 25-30°C for 3-5 days. The yeast species was characterized by observing the colony morphology and selecting colonies for further analysis. Gram staining and sugar tests were also performed to identify the yeast species.

Pretreatment

Collection of waste potatoes: Waste potatoes were collected from markets in Kopargaon and local areas. Washing and cleaning: The potatoes were washed and cleaned to remove dirt and debris. Chopping: 200g of potatoes were chopped into small pieces. Cooking/steaming: The chopped potatoes were cooked/steamed with 400ml of water until they became soft, and the starch was gelatinized. Autoclaving: The gelatinized potato mash was poured into glass flasks and autoclaved for 15-20 minutes at 121°C.

Enzymatic saccharification

For the further breakdown of the starch, enzymatic saccharification occurred. Cooling: The flask containing the potato mash was cooled.

Inoculum addition: 30ml of *Aspergillus niger* inoculum was aseptically inoculated into the potato mash flask at a pH of 5-5.5. Incubation: The flask was incubated at 30-35°C for 48-72 hours with occasional shaking to promote enzyme production and starch breakdown.

Fermentation Process

Yeast inoculum preparation: A 30ml inoculum of *Saccharomyces cerevisiae* was prepared. Inoculum addition: After 72 hours of enzymatic hydrolysis, some of the potato mash flasks were inoculated with the yeast inoculum. pH adjustment: The pH was adjusted to 4.5-5.5 with a buffer solution. Incubation: The flask was incubated at 30-35°C under anaerobic conditions for 3-6 days in a rotary shaker incubator. [18]-[21]

Recovery of Bioethanol

Completion of fermentation: The fermentation process was completed, and the bioethanol was ready for recovery. Collection of bioethanol: The bioethanol was collected from the fermentation vessel.

Distillation Process

Preparation: 410g of the fermented broth containing bioethanol was prepared for distillation by removing any sediment or impurities. Heating: The fermented broth was heated to a temperature of around 75-80°C, which is the boiling point of ethanol. Vaporization: The heat caused the ethanol to vaporize and rise into a condenser. Condensation: The vaporized ethanol was condensed back into a liquid using a condenser. Collection: The condensed ethanol was collected in a separate container, resulting in 30ml of bioethanol. [22]

Analytical testing

Sr. No.	Test	
1	Physical tests	Distillation, Boiling point, Colour, Density
2	Chemical tests	pH, Iodine test, Sulfuric acid test.
3	Spectroscopic tests	IR spectroscopy
4	Biological tests	Antimicrobial activity, Validation

Table No. 2.1 Analytical testing

1. Biological tests

a. Antimicrobial activity

The well diffusion method is based on the principle that the bioethanol will diffuse from the well into the surrounding agar, creating a concentration gradient. The bacteria will grow in the areas where the concentration of bioethanol is low, but will be inhibited from growing in the areas where the concentration of bioethanol is high. The resulting zone of inhibition is a measure of the antimicrobial activity of the bioethanol.

Preparation of agar plate: The Mueller Hinton Agar plate was prepared according to the manufacturer

instructions. Sterilization The agar plate was sterilized by autoclaving at 121°C for 15-20 minutes. Selection of bacterial strain: A 24-hour-old culture of *Pseudomonas spp*. was selected for the antimicrobial activity test. Preparation of bacterial suspension: The bacterial suspension was prepared according to the CLSI (Clinical and Laboratory Standards Institute) guidelines. Inoculation of agar plate: 100 µL of the test sample (bacterial suspension) was spread evenly on the MH agar plate using a sterile spreader. Drying of agar plate: The plate was allowed to dry for 5-10 minutes to prevent the bacterial suspension from spreading. Creation of wells: A cork borer was used to create wells on the agar plate. Addition of bioethanol: Each well was filled with 50-100 µL of bioethanol. Incubation: The plate was incubated at 37°C for 24-48 hours to allow the bacteria to grow and the bioethanol to exert its antimicrobial effect. Measurement of zone of inhibition: The diameter of the zone of inhibition around the well was measured using a ruler or a calipers.

b. Validation test

The validation test is based on the principle that bioethanol will inhibit the growth of *Staphylococcus spp* on Nutrient Agar (NA) plates. The test measures the ability of bioethanol to prevent the growth of *Staphylococcus spp.*

Preparation of bacterial suspension: A bacterial suspension of *Staphylococcus spp* was prepared according to the CLSI guidelines. Preparation of Nutrient Agar (NA) plates: NA plates were prepared and sterilized by autoclaving at 121°C for 15-20 minutes. Inoculation of NA plates: 100 µl of the bacterial suspension was spread evenly across the NA plate using a sterile spreader. Drying of NA plates: The plate was allowed to dry for 5-10 minutes to prevent the bacterial suspension from spreading. Addition of bioethanol: 30-50 ml of bioethanol was spread evenly over the inoculated NA plate. Preparation of positive control plate: A positive control plate with inoculum (without bioethanol) was prepared. Preparation of test plate: A test plate with inoculum and bioethanol was prepared. Incubation: The plates were incubated at 37°C for 24 hours. Observation of colony growth: After 24 hours, colony growth on the test plate was observed. Comparison with positive control plate: The results were compared with the positive control plate to determine the effect of bioethanol on *Staphylococcus spp.*

2. Chemical Test

a. Iodine Test

Based on the reaction between iodine and starch/carbohydrates, which produces a blue-black/ yellow colour.

Purpose: Detected presence/absence of starch/carbohydrates and indicated ethanol presence. Took a test tube with bioethanol sample. Added a crystal of iodine to the sample and the colour change was observed.

b. Sulfuric Acid Test

Based on the dehydration reaction between sulfuric acid and carbohydrates, which produces a coloured compound while negative result shows white precipitation.

Purpose: Detected presence of carbohydrates or sugars. Added sulfuric acid to the bioethanol sample. Observed the sample for colour change or precipitate formation.

3. Spectroscopic tests

a. Infrared Spectroscopy Test

Infrared spectroscopy is based on the principle that molecules absorb infrared radiation at specific wavelengths.

Purpose: Identified the molecular structure of the bioethanol sample Prepared the bioethanol sample by placing it in a sample holder.
Exposed the sample to infrared radiation using an infrared spectrometer. Measured the absorbed or transmitted radiation using a detector. Analyzed the resulting infrared spectrum to identify the molecular structure. [23]-[25]

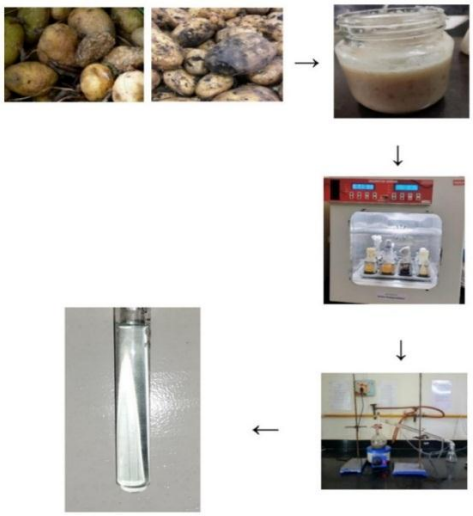

Fig No. 2.1 Overview of Bioethanol Production

RESULT AND DISCUSSION

1. Isolation and Identification of Inoculum

The study successfully isolated *Aspergillus niger* and *Saccharomyces cerevisiae* from their respective sources. *Aspergillus niger* was identified based on its brown-black colonies on PDA plates and confirmed using Lactophenol cotton blue staining, which revealed characteristic fungal structures. The sugar utilization test showed that *Aspergillus niger* could break down sucrose and maltose, indicating its enzymatic activity. Similarly, *Saccharomyces cerevisiae* was identified by its creamy-white colonies on YPD plates and confirmed through Gram staining, which showed its yeast morphology. The sugar test confirmed that it fermented mannitol, sucrose, dextrose, fructose, and maltose, proving its fermentation capability.

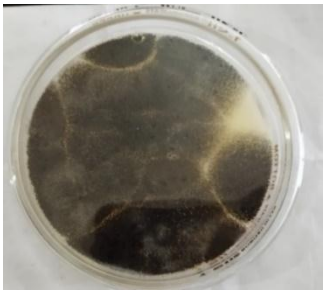

Fig. No. 3.1 *Aspergillus niger* plate

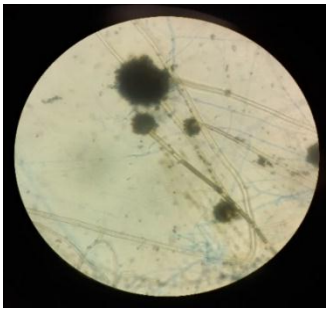

Fig. No. 3.2 Lactophenol cotton blue staining of *Aspergillus niger*

Fig. No. 3.3 *Saccharomyces cerevisiae* growth on YPD Plate

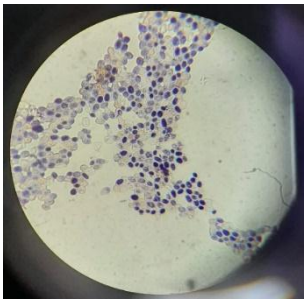

Fig. No. 3.4 Gram staining of *Saccharomyces cerevisiae*

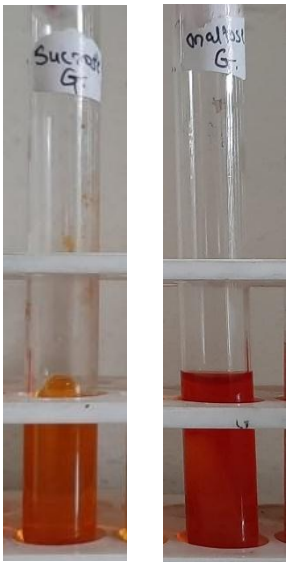

Fig. No. 3.5 Sugar Test (Sucrose, Maltose) For *Aspergillus niger*

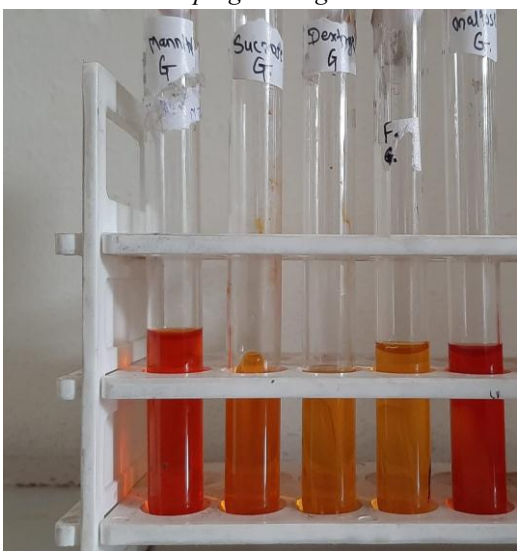

Fig. No. 3.6 Sugar Test (Mannitol, Sucrose, Dextrose, Fructose, Maltose) For *Saccharomyces cerevisiae*

2. Distillation of Bioethanol

The distillation process was conducted at 75–80°C, confirming the presence of ethanol in the fermented broth. The separation of bioethanol from the

fermentation mixture was achieved through distillation, which concentrated the ethanol content. The collected 30 mL of distillate was colourless and exhibited properties consistent with ethanol, making it suitable for biofuel applications. The effectiveness of the distillation step suggested that the fermentation process had successfully produced a significant amount of ethanol. This purified ethanol can be further utilized as biofuel, industrial solvent, or chemical feedstock for various applications.

3. Analytical Tests of Bioethanol

Sr. No	Test Name	Parameter	Result	Standard
1	Distillate	Volume	30 ml	-
2	Colour	Visual	Colourless	Colourless
3	Boiling Point	Temperature	82^0C	82^0C - 83^0C
4	Density	g/cm^3	0.909	0.789
5	pH	Acidity	5.5	4.5-5.5

Table No. 3.1 Analytical Tests of Bioethanol

4. Chemical Tests for Bioethanol Confirmation

The iodine test resulted in a yellow color, confirming the absence of starch and the presence of bioethanol. Similarly, the sulfuric acid test produced white precipitation, indicating the presence of ethanol. These tests verified that the fermentation and distillation processes successfully converted waste potatoes into bioethanol. The absence of starch residues indicated effective enzymatic hydrolysis, while the white precipitate in the sulfuric acid test confirmed ethanol purity. These findings supported the claim that the bioethanol produced was of high quality and could be utilized for fuel and industrial applications.

Fig. No. 3.7 Iodine Test (Yellow Colour)

Fig. No. 3.8 Sulfuric Acid Test (White Precipitation)

5. Biological Test for Bioethanol

The antimicrobial test demonstrated that bioethanol exhibited antibacterial properties against *Pseudomonas spp.* The zone of inhibition was measured at 15 mm and 12 mm, indicating that bioethanol effectively inhibited bacterial growth. The validation test further confirmed that bioethanol effectively suppressed *Staphylococcus spp*., as seen in the comparison with the control plate. These results suggested that bioethanol could potentially be used as a disinfectant or preservative due to its antimicrobial properties. The findings highlighted the potential dual benefits of bioethanol as both a fuel and an antimicrobial agent. [12]-[17]

Fig. No. 3.9 Antimicrobial Test

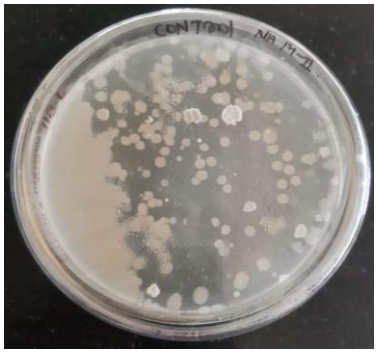

Fig. No. 3.10 NA control plate *staphylococcus spp*

Fig. No. 3.11 NA Test Plate of *staphylococcus spp* for Validation test

6. Infrared (IR) Spectroscopy Analysis

The IR spectroscopy results confirmed the molecular structure of the bioethanol. The presence of functional groups was detected at specific wavenumbers, confirming ethanol chemical composition. [23]-[25]

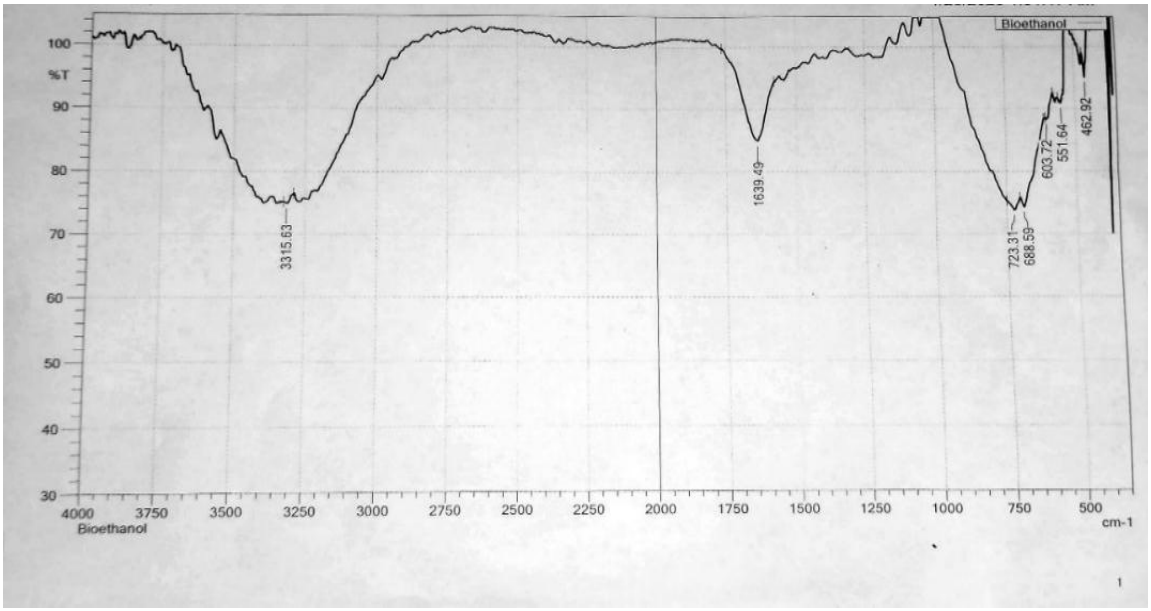

Fig. No. 3.12 Graph of IR Spectroscopy Result

Sr. No.	Wavelength	Bond	Stretching	Result
1	3315.63 cm-1	(O-H)	Hydroxyl group	Presence of alcohol.
2	1639.49 cm-1	(C=C)	Alkene group	Presence of unsaturated hydrocarbons.
3	723.31 cm-1	(C-H)	Alkyl group	Presence of saturated hydrocarbons.
4	603.72 cm-1	(C-O)	Ether group	Presence of oxygen-containing compounds.
5	551.64 cm-1	(C-C)	Alkane group	Presence of saturated hydrocarbons.

Table No. 3.2 Functional Group analysis Table

CONCLUSION

This study demonstrated the successful integration of *Aspergillus niger* and *Saccharomyces cerevisiae* in a co-culture system for bioethanol production from waste potatoes. The use of simultaneous saccharification and fermentation (SSF) improved process efficiency by allowing enzymatic hydrolysis and fermentation to occur simultaneously. Analytical validation confirmed the purity, consistency, and antimicrobial properties of the bioethanol produced, meeting the expected chemical and physical standards.

The findings highlighted the potential of waste potatoes as a sustainable and cost-effective feedstock for bioethanol production. By utilizing agricultural waste, this approach contributed to waste reduction and supported the principles of a circular economy. The co-culture method also enhanced ethanol yield compared to conventional single-microbe fermentation, demonstrating improved substrate utilization and fermentation efficiency.

Furthermore, the antimicrobial properties of the bioethanol suggested additional potential applications beyond fuel production, including its use

as a disinfectant or preservative. The results confirmed that microbial synergy in a co-culture system could enhance bioethanol production while maintaining environmental sustainability.

This study provided a strong foundation for future research into optimizing co-culture fermentation techniques. Further studies could focus on strain improvement, process scalability, and economic feasibility to enhance industrial applications. Overall, the research demonstrated a viable and innovative bioethanol production strategy that aligns with sustainable energy goals and waste management solutions.

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